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Tritrichomonas foetus: induced division synchrony by hydroxyurea

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Abstract Treatment of cultures of *Tritrichomonas foetus* with 4 mM hydroxyurea (HU), a known DNA synthesis inhibitor, induced pseudocyst formation and caused a mitotic burst. An hour after drug release there was a characteristic, synchronous burst of cell division. *T. foetus* culture was arrested in the G2/M phase. The synchrony index varied from 66% to 69%. The synchrony was maintained for several cell cycles, even in thawed cultures which had been frozen for storage in liquid nitrogen. The synchronized cells were analyzed by light and scanning electron microscopy, as well by flow cytometry.

Introduction

Tritrichomonas foetus, a parasitic protist that inhabits the urogenital tract of cattle, presents an unorthodox type of mitosis in which nuclear envelope breakdown does not take place and an extranuclear spindle is present (Brugerolle 1975). There are very few data on this cell division process in trichomonads (Brugerolle 1975; Viscogliosi and Brugerolle 1994; Ribeiro et al. 2000). A major problem in studying the cell division of trichomonads has been the difficulty of obtaining a synchronous culture with a high mitotic index. In the present study we succeeded in synchronizing *T. foetus* cultures by using hydroxyurea (HU), a known inhibitor of DNA synthesis. The synchronized cells were characterized by flow cytometry, and by light and scanning electron microscopy.

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Materials and methods

Organisms and cell culture

The K strain of *T. foetus* has been described previously (Ribeiro et al. 2000). It is maintained in Diamond's TYM medium with 10% FBS (fetal bovine serum) (Diamond 1957).

Synchronization procedure

When cell cultures reached the cell density of 3.0×10^6 cells/ml (approximately 24 h) the drug was added. Different concentrations of HU (Sigma, St. Louis, Mo., USA) were tried (1 mM, 4 mM, 8 mM and 16 mM). The cells were incubated at 36.5°C and the culture analyzed every hour to determine cell density, shape, size, movement, cell cycle phase and general morphology. About 40 triplicate experiments were carried out. After 15 h of incubation in the presence of the drug, the cells were resuspended in fresh medium without the drug. The cultures were maintained during 0–2 h after drug release, observed by video microscopy and then collected for light microscopy staining, scanning electron microscopy and flow cytometry. Several aliquots from synchronized cultures were taken and frozen in liquid nitrogen for storage. Some experiments were undertaken using thawed cultures.

Light microscopy

For light microscopy the cells were fixed in 4% paraformaldehyde, 0.01% glutaraldehyde in warmed PHEM buffer. Samples were counted and stained using the Panotic kit (Laborclin, PR, Brazil). Following treatment with HU , *T. foetus* cultures were evaluated by interferential differential microscopy coupled with a high-resolution video camera (Pasecon-Pal-G system/Grundig electronic or NTSC-optronics camera) and with the digital image processing system IBAS (Kontron-Zeiss) attached to the microscope.

Scanning electron microscopy

Untreated cells and cells with under 15 h drug treatment and 1 h release were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, washed in PBS, post-fixed in 1% OsO_4 in 0.1 M cacodylate buffer, dehydrated in ethanol, critical point dried, and observed in a Jeol 5800 scanning electron microscope.

Mitotic index

The mitotic index was calculated as follows:

$$\mathrm{MI} = \frac{\text{Dividing cells}}{\text{Total of counted cells}} \times 100 \qquad \text{(a)}$$

Synchrony index

The two synchrony indexes was calculated using the following formulas: (1) F: $(N/N_0{-}1){-}(2^{t/g}{-}1)$ (Blumenthal and Zaler 1962), where N = final number of cells, N_0 = initial number of cells, t = time interval, and g = generation time and (2) F: $T/2{-}D_{1/2}$: $T/2$ (Rueckert and Mueller 1960), where T = generation time, and $D_{1/2}$ = time for half of the cells to divide.

Flow cytometry

The cells were centrifuged and resuspended in PBS containing 10 μg/ml ethidium bromide (EB) and 0.05% Triton X-100 (Sigma). Analyses were performed using a Coulter Elite Flow Cytometer (Coulter, Hialeah, Fla.). A forward scatter versus side scatter plot of 488 nm argon laser deflected light was used to set the size and granularity of the parasite population and the gate of analysis. Data was acquired using peak pulse and integral pulse together in order produce the gate doublets. Analysis was based on the accumulation of 10^4 cells.

Results and discussion

Previous studies have shown that even at high concentrations (15 mM) HU did not have a detectable effect on the *Trichomonas vaginalis* cell cycle (Riley et al. 1994). We determined the minimum concentration of HU required for the trichomonads to traverse their cell cycle reaching a blockage point with no apparent toxicity to the majority of cells (Figs. 1, 2). At a concentration of 16 mM HU, the treatment induced the appearance of pseudocysts and the culture presented multinuclear cells. In addition, the cells showed slower movement. With 1 mM HU there was no detectable effect (data not shown). We observed a growth inhibition of about 50% when 8 mM HU was used. HU at a concentration of 4 mM was selected since the cell number was only slightly below the untreated culture control (Fig. 1) and seemed to cause less alteration in cell shape and movement when compared to the 8 and 16 mM concentrations.

When cells treated with 4 mM HU were released from the drug by washing in fresh medium, an initial lag period occurred, lasting approximately 1 h, in which the culture had few forms in division. Afterwards, a burst of synchronous cell division occurred. The culture exhibited actively living cells with rapidly beating flagella, as shown by video microscopy, but the cells were larger and bulkier than the control (Fig. 3). Thereafter, a period of rapid primary growth was observed during which the culture population increased by approximately 44% in 6 h. Following this synchronous burst, the rate of growth returned to a level

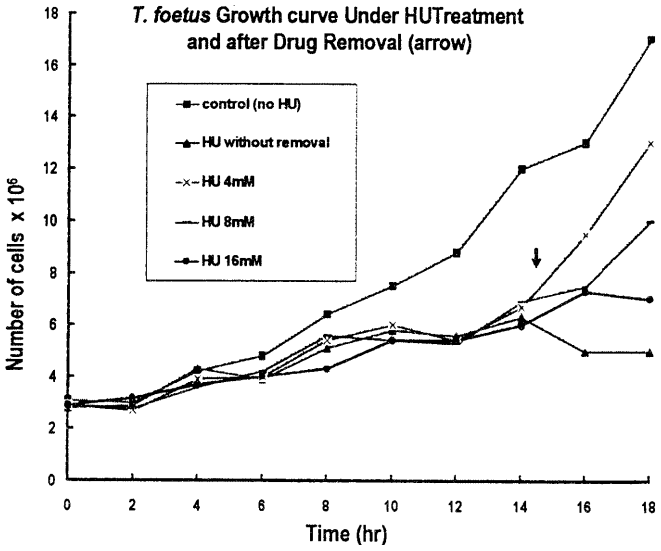

Fig. 1 Effects of hydroxyurea (HU) on the growth of *Tritrichomonas foetus*. Different concentrations of the drug (16 mM, 8 mM, 4 mM) were added to exponentially growing cultures and incubation continued at 36.5°C. The *arrow* points to the moment of drug release. Controls: (–) culture in complete medium without drug or (+) with HU 16 mM throughout the experiment. The effects of HU on the growth of *T. foetus* were repeated ten times with reproducible results and the data shown are from representative experiments

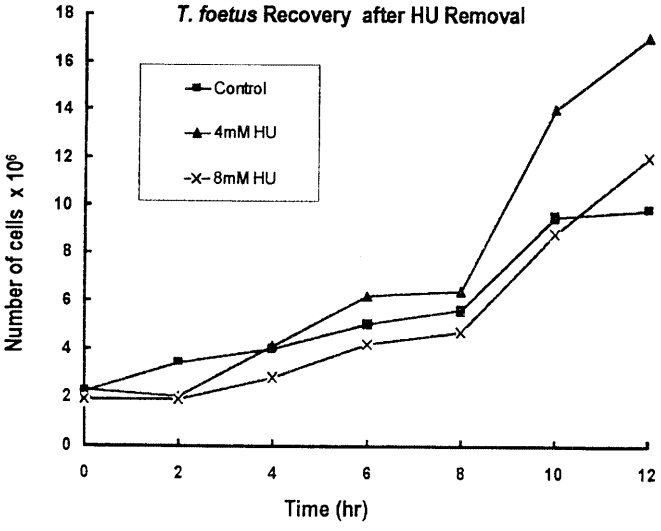

Fig. 2 *T. foetus* cell growth monitored after HU removal. Cells were incubated in HU for 15 h in the culture medium, washed twice in fresh medium and then resuspended in conditioned medium without the drug. In the control, no drug was added. Notice that the cells previously treated with 4 mM HU showed a burst of growth after 8 h of drug removal

Fig. 3 General view of *T. foetus* culture by scanning electron microscopy after 1 h release from 4 mM HU after 15 h treatment. Many cells show signs of division (*arrowheads*). ×5,000

slightly lower than that observed for the untreated cells (not shown). Some synchronized cell cultures were frozen in liquid nitrogen and thawed 1 week later. The synchrony index was maintained for at least 30 h (not shown).

The synchrony indexes of Blumenthal and Zaler (1962) and Rueckert and Mueller (1960) were found to be 69% and 66.6%, respectively. Quantitative analysis of HU treated cultures showed a significant increase in the percentage of mitotic cells. The untreated culture, analyzed by light, scanning and video microscopy, presented only 22.26% of cells in mitosis whereas after the HU 4 mM release it reached 40.04% (Table 1). The mitotic index was maintained for several cell passages (not shown). Prolonged periods of culture gradually decreased this proportion. Cell cultures which were frozen for storage 5 h after drug removal maintained their synchrony after recovery. It seemed that the cultures were arrested at the G2/M boundary, since at least 50% of the cells remained in the pre-mitotic phase. This was determined by the duplication of the axostyle, costa, and flagella, but not the nucleus.

DNA content analysis by flow cytometry

Figure 2 shows the number of cells and relative amount of DNA in linear and logarithmic scales in cells with and without HU treatment. A distinct peak of lower DNA content and a shift of about 20% of the cell population to this histogram region was observed throughout the treatments. A partial shift of the cell population to the G1/S phase of the cell cycle was observed, as is the case in higher eukaryotes. Furthermore, flow cytometry showed that most of treated cells were distributed at a higher and heterogeneous DNA content region of the linear fluorescence histograms, suggesting normal DNA synthesis (Fig. 4).

Effect of HU on the morphology of *T. foetus*

T. foetus which underwent a 4 mM HU treatment were observed in a slow motion video. They exhibited actively beating flagella, although some treated cells were rounded and presented reduced flagellar oscillations. The normal culture (control) presented only 22% of cells in mitosis, 73% in interphase and about 4% as pseudocysts. Pseudocysts are forms which are generally spherical, lack a true cyst wall and present internalized flagella (Granger et al. 2000) (Fig. 3). Multinucleated cells were frequently found (Fig. 4). With higher doses of HU (8 mM and 16 mM) or prolonged incubation (over 24 h), we found a dramatic decrease in cell movement, and the appearance of a number of pseudocysts (Table 1). When 4 mM HU was used, pseudocysts were observed more frequently (19.13%) than in the controls (3.96%). Figure 3 shows a general view of the culture under treatment with 4 mM HU for 15 h, followed by incubation in fresh medium after drug release for 2 h. Scanning electron microscopy revealed a large number of cells in mitosis as well some pseudocysts. Figure 5 shows that 40% of the culture was in mitosis 6 h after drug removal.

The establishment of a protocol to obtain synchronized cultures of *T. foetus* represents a preliminary step necessary for the further investigation of the division

Table 1 Morphology of *Tritrichomonas foetus* by scanning electron microscopy and the mitotic index. Analysis of *T. foetus* cultures in controls and with hydroxyurea (*HU*) treatment at different concentrations and after drug removal. The percentage of dividing forms and cells in interphase are given. The percentage of *T. foetus* cells under different conditions: control and after drug treatment as observed by scanning electron microscopy

	Pseudocysts	Interphase	Mitosis	Total cell number
Control (no drug)	3.96%	73.78%	22.26%	328
4 mM HU (15 h incubation with 6 h release)	19.13%	40.83%	40.04%	507
4 mM HU (15 h incubation with no release)	30.99%	36.31%	32.70%	826
20 mM HU (15 h incubation with no release)	22.90%	49.17%	28.74%	602

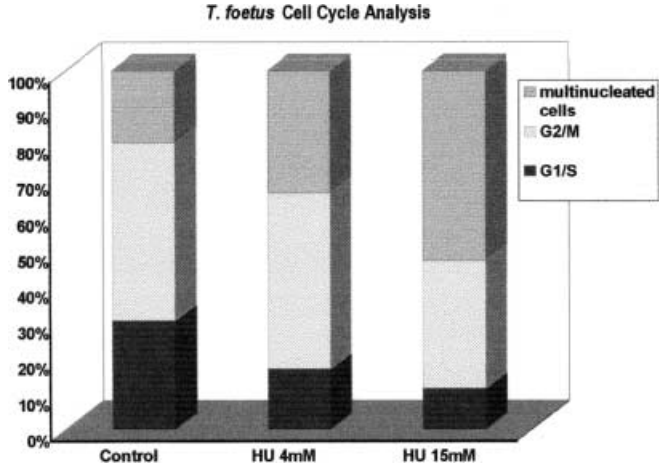

Fig. 4 *T. foetus* incubated with HU 4 mM for 15 h. After drug removal the cells were counted every 2 h. Control cells were not incubated with HU. Notice that 40% of the culture is in mitosis 6 h after drug removal

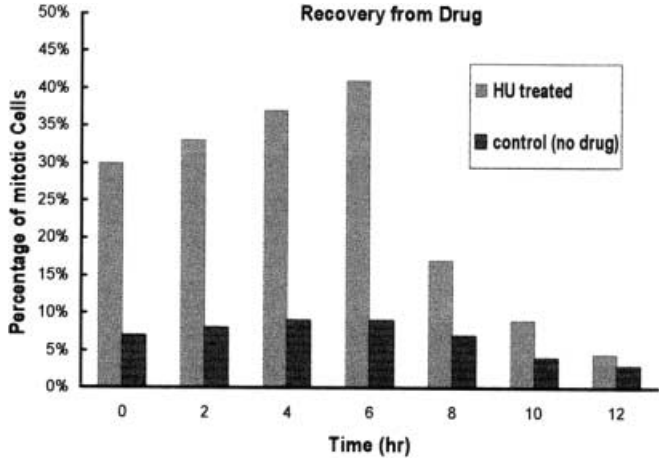

Fig. 5 *T. foetus* DNA content during cell cycle phases. After 15 h of HU incubation the cells were processed for flow-cytometry. Control cells were not incubated with HU. Notice that the G1/S and G2/M phases decrease at higher drug concentrations whereas the multinucleated cell population increases

process and the cell cycle of this protist. In *Crithidia fasciculata,* HU inhibits cell growth resulting in a 70% synchrony upon its removal (Cosgrove et al. 1979). In yeast and in vertebrates, treatment with HU arrests cells at the G1/S phase boundary or within the S phase (Sinclair 1967; Young et al. 1967; Pardee et al. 1978; Ikegami 1978). Treatment with 10 mM HU gave an 85% synchrony index in *Entamoeba histolytica* (Austin and Warren 1983). In *Trypanosoma brucei* HU (7.5 μg/ml) inhibited cell division but did not inhibit DNA synthesis. Most of the cells were arrested in the G2/M phase of the cell cycle, with all cells presenting two kinetoplasts but only one nucleus (Mutomba and Wang 1996). In *Leishmania tarentolae* (Simpson and Braly 1970), a 41% synchrony index was achieved with HU, but synchronization decayed after 2 cycles.

Previous studies have shown that even a high concentration (15 mM) of HU did not have a detectable effect on the *Tric. vaginalis* cell cycle (Riley et al. 1994). These authors claimed that *Tric. vaginalis* cells lack ribonucleotide reductase (Wang and Chen 1984) and are incapable of reducing ribonucleotides to deoxyribonucleotides, observations that could explain the relative insensitivity of *T. vaginalis* to HU (Riley et al. 1994). Our findings for *T. foetus* are in disagreement with these authors. This could be either because we used another species or because we carried out counting using images under scanning and light microscopy while they used flow cytometry. On the other hand, Riley et al. claimed that *Tric. vaginalis* remained motile while we found a dramatically reduced flagellar movement in *T. foetus* when the same concentrations were used. Our observations show that *T. foetus* is sensitive to HU, suggesting that: (1) it could have a ribonucleotide reductase and, therefore, the capacity to synthesize nucleotides (Mutomba and Wang 1996), or (2) HU action in *T. foetus* could be provoked by a different mechanism which has not yet been elucidated.

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